The findings in the experimental neuropathy consisted of axonal swelling with thinning of the myelin sheath or denudation of myelin near the node of Ranvier associated with accumulated masses of neurofilaments. This type of abnormality is very similar to the giant axonal change described by Asbury *et al.* (*4*) in a sporadic case of a slowly progressive mixed polyneuropathy without known exposure to toxins. It is also of interest that the peripheral nerve can show neurofilamentous alterations with other agents including acrylamide (*5*), β,β'-iminodipropionitrile (*6*), and vincristine (*7*) but none are identical to those described here with MBK or in the case of the giant axonal neuropathy. It is also important that in our experimental neuropathy focal areas of denudation of myelin were present without swelling of the axon. This change could be secondary to axonal damage, although we cannot rule out the possibility of direct toxicity of MBK for the myelin sheath.

Safe atmospheric levels of MBK should be established; it is also necessary to ascertain whether the recommended threshold limit value of 100 ppm for MBK provides an adequate margin of safety for workers exposed to this solvent.

Note added in proof: After this manuscript was submitted for publication, Spencer and Schaumberg (*8*) reported similar findings in rats exposed to MBK at 1300 ppm, 6 hours per day, 5 days a week, for up to 4 months.

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References and Notes

1. N. Allen, J. R. Mendell, D. Billamier, R. Fontaine, *Trans. Am. Neurol. Assoc.*, in press.
2. M. J. Karnovsky, *J. Cell Biol.* **27**, 137A (1965).
3. P. J. Dyck, J. A. Gutrecht, J. A. Bastron, W. E. Karnes, A. J. D. Dale, *Mayo Clin. Proc.* **43**, 81 (1968).
4. A. K. Asbury, M. K. Gale, S. C. Cox, J. R. Baringer, B. O. Berg, *Acta Neuropathol.* **20**, 237 (1972).
5. J. B. Prineas, *J. Neuropathol. Exp. Neurol.* **28**, 598 (1969).
6. S.-M. Chou and H. A. Hartman, *Acta Neuropathol.* **4**, 590 (1965).
7. S. S. Schochet, Jr., P. W. Lampert, K. M. Earle, *J. Neuropathol. Exp. Neurol.* **27**, 645 (1968).
8. P. S. Spencer and H. H. Schaumberg, paper presented to the American Association of Neuropathologists, Boston, Mass., 7 to 9 June 1974.
9. Supported by Borden Chemical Division of Borden, Inc., Columbus, Ohio, and the Bremer Foundation, Ohio State University, Columbus.

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Complement-Dependent Stimulation of Prostaglandin Synthesis and Bone Resorption

Abstract. *Complement-sufficient heterologous serum induced prostaglandin synthesis and resultant resorption in cultures of fetal rat long bones. Bone resorption was enhanced with unheated normal rabbit serum as compared to heated serum or serum from rabbits lacking the sixth component of complement (C6). Addition of functionally purified C6 restored resorptive activity in C6-deficient serum. Concentrations of prostaglandin E were increased in the culture media of bones incubated with complement-sufficient serum. The resorptive effects of active serum as well as the appearance of prostaglandin E in the media were inhibited by indomethacin.*

The resorption of fetal bone in organ culture can be stimulated by the addition of serum to the medium (*1*). Histological evidence for the role of complement (C) in the breakdown of cartilage matrix, impaired growth, and increased bone resorption in cultures of mouse and chick bone rudiments containing serum was obtained by Fell, Lachmann, Coombs, Dingle, and Weiss (*2*). The destructive effects on cartilage probably required the presence of connective tissue cells (*3*). These investigators attributed the observed effects to immune activation of C, since antiserum to cell surface antigens or to bone tissue intensified the activity and depletion of C from serum or the use of C-deficient serum abrogated the response. Complement activation increased the release of lysosomal enzymes in their cultures.

We have assessed the role of C in stimulation of bone resorption by serum, using a quantitative assay based on the release of previously incorporated radiocalcium from fetal rat bone in organ culture (*4*). Nineteen-day, fetal rat long bone shafts (radius and ulna) previously labeled with ^{45}Ca, were cultured in modified BGJ medium; 50 percent rabbit serum served as the source of C. The bones were cultured for 6 days, with one medium change at 3 days. Paired bones were used for control and experimental cultures.

Serum from ten normal rabbits all showed heat-labile stimulation of bone resorption (Table 1) and only 1 of 16 individual assays failed to show a significant difference between experimental and control cultures after 6 days. The mean increase in release of ^{45}Ca was approximately 60 percent and most of this occurred during the second 3 days in culture. When an increase in release of ^{45}Ca was observed during the first 3 days, it was usually less than 30 percent. The experimental-to-control ratios achieved were not as high as those attained with other experimental models that employed parathyroid hormone or osteoclast-activating factor from human leukocytes (*4*, *5*), largely because (i) no preculture was used to remove exchangeable ^{45}Ca and (ii) a relatively high rate of control resorption was produced by the presence of 50 percent heated serum in the medium. At the end of 6 days, bones cultured in unheated serum showed marked histologic change; compared with bones cultured in heated serum, there was loss of matrix and proliferation of osteoclasts and

Table 1. Bone-resorbing activity of rabbit serum. Values are means ± standard errors for number of assays given in parentheses; each assay consisted of four pairs of bones cultured with the indicated serum. C6, the sixth component of complement.

Rabbit serum		6-day ^{45}Ca release (experimental/control ratio)
Experimental	Control	
Normal, unheated	Normal, heated*	1.59 ± 0.06† (16)
C6-deficient, unheated	C6-deficient, heated*	1.05 ± 0.05 (14)
C6-deficient plus human C6, unheated	C6-deficient plus human C6, heated*	1.41 ± 0.11† (4)
C6-deficient plus guinea pig C6, unheated	C6-deficient plus guinea pig C6, heated*	1.44 ± 0.15† (4)
Normal plus human C6	Normal	1.05 ± 0.02 (3)
Normal plus guinea pig C6	Normal	1.14 ± 0.02† (3)
Normal plus guinea pig C2	Normal	0.94 ± 0.13 (1)
C6-deficient plus guinea pig C2	C6-deficient	0.98 ± 0.23 (1)
Human C6	Vehicle	1.02 ± 0.05 (1)
Guinea pig C6	Vehicle	1.09 ± 0.01† (1)

* 56°C, 30 minutes. † Significantly greater than 1.0, $P < .05$.

Table 2. Prostaglandin E (PGE) content of media after 6 days of culturing with various rabbit serums and with indomethacin ($10^{-5}M$). Values are means ± standard errors for the indicated number of cultures, each containing four bones per milliliter of medium.

Serum	Cultures (No.)	6-day ^{45}Ca release (count/min per milliliter)	Medium PGE (nanograms per culture)
Heated normal*	6	2280 ± 160	0.1 ± 0.1
Unheated			
Normal	8	3390 ± 220†	3.3 ± 0.4†
Normal plus indomethacin	8	1860 ± 170	1.0 ± 0.6
C6-deficient	7	1990 ± 120	0.8 ± 0.5
C6-deficient plus guinea pig C6	7	2970 ± 190‡	4.0 ± 1.2‡
C6-deficient plus guinea pig C6 plus indomethacin	7	1760 ± 230	0.6 ± 0.5

* 56°C, 30 minutes. † Significantly different from heated normal serum, $P < .05$. ‡ Significantly different from C6-deficient serum, $P < .05$.

fibroblasts but no extensive cell necrosis. To identify the heat-labile activity as C, serum from rabbits deficient in the sixth component of C (C6) (*6*) was used. The mean ratio of the release of ^{45}Ca in cultures containing unheated C6-deficient serum was not significantly different from those containing heated C6-deficient serum (Table 1). Only 2 of 14 assays from nine rabbits showed significant resorption, and in these release of ^{45}Ca was less than 30 percent above the control. Functionally purified human or guinea pig C6 (*7*) significantly restored the bone-resorbing activity to C6-deficient serum (Table 1). Human C6 added to normal rabbit serum had no stimulatory effect. Guinea pig C6 caused a small (9 to 14 percent) increase in release of ^{45}Ca when added alone or to normal serum. Addition of C2 was without effect.

The delayed stimulation of resorption by C-sufficient serum in this system suggested that synthesis of a mediator by the bone tissue itself might be involved. Since prostaglandins are potent stimulators of bone resorption, which produce a slower response than such agents as parathyroid hormone or active vitamin D metabolites (*8*) and are synthesized in many tissues, the possibility that they mediated the serum effect was examined next. Indomethacin has been shown to inhibit prostaglandin synthesis (*9*). The addition of this agent at $10^{-5}M$ to bone cultures inhibited the resorbing activity of normal rabbit serum as well as that of C6-deficient serum supplemented with C6 (Table 2). Indomethacin did not block the response to parathyroid hormone or exogenous prostaglandin E_2 (PGE_2) (Fig. 1).

Media from these cultures were assayed for prostaglandin E (PGE) content by a specific radioimmunoassay procedure (*10*). Bones cultured with heated normal serum released little PGE into the medium (Table 2). However, bones cultured with unheated normal serum showed not only increased release of ^{45}Ca but also a marked increase in PGE in the medium. Indomethacin inhibited the synthesis and release of PGE into the culture medium as well as release of ^{45}Ca from the bone. The addition of C6 to C6-deficient serum resulted in a fivefold increase in the PGE content of the medium, associated with a 50 percent increase in the release of ^{45}Ca. Pooled extracts from bones cultured in unheated normal serum, which showed the greatest resorption, contained detectable PGE but only about 10 percent of the amount in the culture medium. Prostaglandin E was not detectable in extracts of bones cultured in heated or C6-deficient serum. When media from the first and second 3-day periods were compared for PGE content, more than 80 percent of the total was found in the medium from the second period, indicating delayed synthesis and release from the bone. The concentrations of PGE in the medium in the second period were as high as $4 \times 10^{-8}M$. Since PGE produced by the bones would be diluted upon entering the medium, the concentrations at the bone resorbing sites were probably much higher. Nevertheless, the addition of this amount of PGE_1 or PGE_2 can cause significant stimulation of resorption in cultured bone (*8*). The ^{45}Ca release experimental-to-control ratios of 1.45 and 1.71 were obtained when PGE_2, at concentrations of $10^{-8}M$ and $10^{-7}M$, respectively, was added to the cultures.

These results demonstrate the involvement of C in the resorption of bone and, furthermore, attribute this effect to the enhanced synthesis of prostaglandin by the bone. Activation of C components, at least through C6, was required for this response.

Results of previous work have suggested that antibody to cell surface antigens activates C on the cell membrane and initiates cartilage breakdown (*2*). The rabbit serum we used probably contained "natural" antibodies to some cell surface component of the fetal bone explants since we found

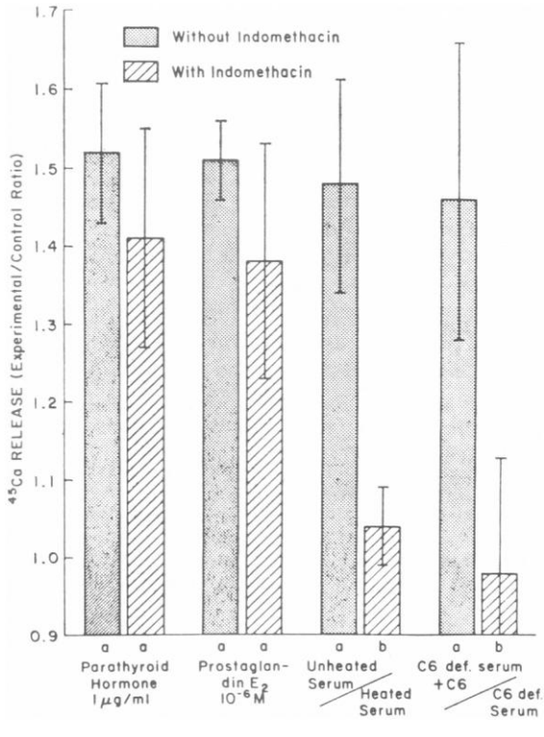

Fig. 1. The effect of indomethacin ($10^{-5}M$) on bone resorption produced by various stimulators. Columns indicate the mean, and vertical lines the standard error for four pairs of cultures. (a) Experimental/control ratio significantly greater than 1.0 ($P < .05$); (b) experimental/control ratio not significantly greater than 1.0. Abbreviation: *def.*, deficient.

hemagglutinating antibodies for rat erythrocytes at the serum concentrations used in our studies. Additional studies (*11*) have shown marked enhancement of C-dependent bone resorption by antibodies obtained by immunization of rabbits with rat erythrocytes or rat bone sonicates. However, non-tissue-related antigen-antibody complexes, which activate either the classical or alternate C pathways (*12*), were ineffective in enhancing the release of ^{45}Ca in cultures containing C-sufficient serum. Moreover, preparations containing the active complement fragments C3a and C5a did not enhance bone resorption (*13*), which is not surprising in view of the C6 requirement for the effect. These findings all indicate that C activation must take place on a cell membrane.

The mechanism by which C activation increases PGE synthesis is not known. Conceivably, alteration of the cell membrane by C activation might provide a signal for increased PGE synthesis. Alternatively, C activation on a membrane might result in the release of fatty acids from membrane phospholipids which could then serve as precursors of prostaglandins. Fatty acids may also stimulate bone resorption directly but only at concentrations of $10^{-4}M$ or higher (*14*).

Prostaglandins have been detected in inflamed gingival tissue and exudates as well as in supernatants of rheumatoid synovial cultures (*15*). The present studies indicating a relationship between C activation and PGE synthesis could help explain these findings as well as the associated pathologic breakdown of adjacent bone in such disorders as rheumatoid arthritis and periodontal disease.

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References and Notes

1. P. H. Stern and L. G. Raisz, *Exp. Cell Res.* **46**, 106 (1967); E. Hausman, R. Genco, N. Weinfeld, R. Sacco, *Calcif. Tissue Res.*, in press.
2. H. B. Fell and L. Weiss, *J. Exp. Med.* **121**, 551 (1965); H. B. Fell, R. R. A. Coombs, J. T. Dingle, *Int. Arch. Allergy Appl. Immunol.* **30**, 146 (1966); P. J. Lachmann, R. R. A. Coombs, H. B. Fell, J. T. Dingle, *ibid.* **36**, 469 (1969).
3. H. B. Fell and M. E. J. Barratt, *Int. Arch. Allergy Appl. Immunol.* **44**, 441 (1973).
4. L. G. Raisz, *J. Clin. Invest.* **44**, 103 (1965); ——— and I. Niemann, *Endocrinology* **85**, 446 (1969).
5. J. E. Horton, L. G. Raisz, H. A. Simmons, J. J. Oppenheim, S. E. Mergenhagen, *Science* **177**, 793 (1972).
6. U. Rother and K. Rother, *Z. Immunitaetsforsch. Exp. Ther.* **121**, 224 (1961).
7. Functionally purified human and guinea pig C6 and guinea pig C2 were obtained from the Cordis Company together with appropriate vehicles containing gelatin. These preparations were reconstituted, dialyzed against BGJ, and added to the culture medium at a final concentration of 25 percent.
8. D. C. Klein and L. G. Raisz, *Endocrinology* **86**, 1436 (19 → A. H. Tashjian, E. F. Voelkel, L. Levine, P. Goldhaber, *J. Exp. Med.* **136**, 1329 (1972); J. M. Goodson and L. G. Raisz, unpublished observations.
9. J. R. Vane, *Nat. New Biol.* **231**, 232 (1971).
10. Media were acidified with citric acid and extracted with ethyl acetate; the extracts dried under nitrogen. Bones were extracted with ethyl alcohol. Samples were then dissolved in benzene–ethyl acetate (60 : 40) and chromatographically purified by the method of R. M. Zusman, B. V. Caldwell, L. Speroff [*Prostaglandins* **2**, 41 (1972)], which separates PGE from fatty acids, PGA, PGB, and PGF (prostaglandins A, B, and F, respectively). After conversion of the PGE to PGB by base hydrolysis, the samples were assayed with a commercial antibody to PGB_2 according to the method of L. Levine and H. Van Vunakis [*Biochem. Biophys. Res. Commun.* **41**, 1171 (1970)]. This assay measures both PGE_1 and PGE_2, but was expressed in terms of PGE_2 equivalents uncorrected for recovery (recovery of PGE_2 averaged 55 percent).
11. A. L. Sandberg and L. G. Raisz, in preparation.
12. A. L. Sandberg, A. G. Osler, H. S. Shin, B. Oliveira, *J. Immunol.* **104**, 329 (1970); A. L. Sandberg and A. G. Osler, *ibid.* **107**, 1268 (1971).
13. L. G. Raisz, R. A. Snyderman, S. E. Mergenhagen, unpublished observations.
14. B. Samuelsson, E. Granstrom, K. Green, M. Hamberg, *Ann. N.Y. Acad. Sci.* **180**, 138 (1971); P. H. Stern, *Experientia* **27**, 1061 (1971).
15. J. M. Goodson, F. Dewhirst, A. Brunetti, *J. Dent. Res.* **52**, 182 (1973) (special issue); D. R. Robinson, H. Smith, L. Levine, *Arthritis Rheum.* **16**, 129 (1973).
16. Supported by PHS grants AM 05205 and DE 03556.
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Selective Alkylation: A Biomimetic Reaction of the Antileukemic Triptolides?

Abstract. *The potent antileukemic plant principles triptolide and tripdiolide contain a characteristic hydrogen-bonded 9,11-epoxy-14β-hydroxy system. They alkylate propanethiol in a process which involves opening of the epoxide function with neighboring hydroxyl assistance. The reaction may mimic the inhibition of tumor growth via selective alkylation of the thiol groups of key enzymes concerned with growth regulation.*

Triptolide (**1**) and tripdiolide (**2**) have recently been characterized as the novel and highly active antileukemic principles of the plant *Tripterygium wilfordii* Hook (*1*). The compounds at 0.1 mg/kg show impressive life-prolonging effects (that is, $T/C \geq 230$) in mice afflicted with the L-1210 lymphoid leukemia (*2*). Biological and chemical data are presented in support of the importance of intramolecular catalysis (by a neighboring hydroxy group on the opening of an epoxide by nucleophiles) for the mode of action of the antileukemic triptolides. The hypothesis is discussed in light of earlier proposals that other plant-derived tumor inhibitors may act via selective alkylation of the thiol groups of key enzymes concerned with growth regulation (*3–7*).

The nuclear magnetic resonance (NMR) spectra of the antileukemic triptolides **1** and **2** display resonances at τ 7.16 (doublet, $J = 11$ hertz, 14-OH, disappears upon D_2O addition) and τ 6.50 (doublet of doublets, $J = 11$ hertz and $J = 1$ hertz, 14-H, collapses to a singlet upon D_2O addition). The highly distinctive J_{HCOH} coupling constant (11 hertz) is attributable to the rigid *trans* orientation of the coupled protons resulting from strong hydrogen bonding between the 14-hydroxyl and the 9,11-epoxide groups (*8*). Free rotation J_{HCOH} coupling constants are typically 3 to 6 hertz (*9*). The co-occurring triptolide **3** ("triptonide") differs structurally from **1** solely at C-14, which bears a ketonic function rather than a β-oriented hydroxyl. Accordingly, the NMR spectrum of **3** does not show the resonances attributable to the hydrogen-bonded hydroxy-epoxide system. Triptonide shows no antileukemic activity in doses up to 0.4 mg/kg. These facts led us to hypothesize that the 9,11-epoxy-14β-hydroxy system is necessary for the antileukemic activity of the triptolides. Furthermore, intramolecular catalysis by the 14-hydroxyl group may assist selective alkylation of biological macromolecules by the 9,11-epoxide. Subsequent testing of the minor variants 14-epitriptolide [**4**, with